

CONTROL OF THE CIRCADIAN RHYTHM OF ACTIVITY IN THE COCKROACH

III. A POSSIBLE ROLE OF THE BLOOD-ELECTROLYTES

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INTRODUCTION

The physiology of the circadian rhythm of locomotor activity in the cockroach has been the subject of investigations by Harker (e.g. 1964), Roberts (e.g. 1966) and Brady (e.g. 1967*a*), but many questions remain unanswered. It appears probable that the rhythm is controlled at some level by blood-borne factors, but it is not clear whether these are hormonal or not.

Control of the activity rhythm in the cockroach requires that locomotor movements be suppressed during daylight, released at dusk and maintained in the released state for the first few hours of darkness. The rhythm is truly circadian, since it is maintained at *circa* 24 hr. under constant environmental conditions. There must therefore be a 'clock' timing the suppression and release of activity in the absence of environmental cues. This timing mechanism appears to be physiologically distinct from the suppression-release mechanism since it can be shown that covert periodicity is maintained in an animal which shows no overt activity rhythm (Harker, 1964; Brady, 1967*a*). On the assumption that the two problems may therefore be studied independently this paper is concerned with an investigation into the suppression-release mechanism only.

Two clues were suggested by the literature. First, the work of Hoyle (1954) and Ellis & Hoyle (1954) indicates that the activity level of the locust may be controlled by the concentration of potassium in its blood. Secondly, a brief report by Pichon & Boistel (1963) contains suggestive evidence that peak activity (i.e. the rhythmically expressed part of activity) in the cockroach locomotor cycle is eliminated by a high-potassium diet.

Hoyle (1954) states that tonus is maintained in *Locusta* by a background of impulses from the 'slow' fibres, and that occasional 'fast' fibre impulses are responsible for reflex and spontaneous movements. He argues that high blood potassium concentrations, by lowering the resting and action potentials, must lower the muscles' capacity for contraction, leading to reduced proprioceptive feed-back to the central nervous system, and hence to decreased tonus and spontaneous activity. High blood potassium levels occur in the feeding locust whose spontaneous activity is thereby reduced thus tending to keep the animal on its food plant; the converse applies in the starving locust which has a low potassium concentration tending to raise its level of spontaneous movements thereby helping it to find new food.

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If cockroach neuromuscular physiology functions similarly it is possible that the same principles may be effective in regulating its activity cycle. If a fall in blood potassium occurs at dusk, Hoyle's argument would indicate that an increase in spontaneous activity might result and that the circadian activity peak might thus be triggered off.

METHODS

The *Periplaneta americana* used in this study were reared in a large tank at about 25° C. and provided with whole crushed oats, apple and water *ad libitum*. A 12 hr. light : 12 hr. dark régime (LD 12:12) was maintained in the tank with the change from light to dark (LD transition) occurring at 12.00 hr. G.M.T.

Four batches of twenty to thirty healthy, young, male cockroaches were selected for experiment. Each batch was placed in a large glass jar (c. 4 litres capacity) and kept in an incubator at constant temperature ($\pm 0.5^\circ$ C.) under the same LD cycle as before. After placing a batch in an incubator at least 2 weeks were allowed to elapse before blood samples were taken. It is known that virtually all *Periplaneta* respond to LD 12:12 by showing a clear 24 hr. rhythm in their activity (Brady, 1967*b*), but for confirmation cockroaches were frequently sampled from each batch and their rhythmicity tested in actographs (Brady, 1967*a*).

For ion analysis a blood sample of about 5 μ l. was collected into a micro-pipette from the meta-coxal membrane of a hand-held cockroach. Collection of blood from a time-sample of five cockroaches was completed in less than 10 min. When samples were taken during the dark-phase of the LD cycle the individuals were removed from their incubator in the dark, but the light was switched on briefly during the time necessary to collect the blood. When pairs of blood samples were taken from a single individual, for matched haemocyte counts and ion analysis, the procedure described earlier (Brady, 1967*d*) was followed.

Blood for ion analysis was expelled from the micro-pipette into 10 ml. of distilled water. Determinations were performed on a Unicam SP 900 flame-photometer against standard solutions of KCl and NaCl, to an estimated accuracy of ± 0.3 mM K⁺/l. and ± 5.0 mM Na⁺/l. Details of the techniques and precautions followed have been described elsewhere (Brady, 1967*d*).

RESULTS

Eight series of samples were analysed from the four batches of cockroaches, making a total of 166 individual blood samples. The animals in batch A were sampled four times and in batch B twice. In each series cockroaches were selected from the batch in groups of 3-6 animals (most groups were of 4 or 5) to make up 4-6 time-samples spanning the period from 3 hr. before the LD transition to 7 hr. after it. Peak activity in *Periplaneta* normally begins during the first hour of darkness and lasts for 1-3 hr. The range of times selected should therefore have covered all the likely physiological changes immediately associated with the onset, expression and decline of activity.

The results of the analyses are summarized in Table 1, which shows the mean concentration of potassium ions in the blood of the animals comprising each time-sample (the specimens being analysed individually, i.e. not pooled). It is apparent that considerable fluctuations occur, both within a single series of time-samples and between

Table 1. *Potassium concentration in the blood of Periplaneta sampled at different times of day*

Series no.	Batch	Date sampled	No. of animals sampled	Time span of samples, G.M.T. (hr.)												Over-all mean K ⁺ concentration
				09.05 -09.45	09.55 -10.25	10.40 -11.20	11.25 -11.55	12.35 -13.10	13.15 -13.45	13.50 -14.15	16.00 -16.15	18.30 -19.15				
1	A	10 Sept.	25	12.3	—	—	10.9	8.1	11.3	—	—	—	—	—	10.1	10.6
2	A	19 Oct.	15	—	—	10.6	10.1	7.4	—	—	8.2	—	—	—	—	9.1
3	A	29 Oct.	16	—	—	11.6	—	—	{9.1 9.9	—	8.4	—	—	—	—	9.7
4	A	9 Nov.	17	—	10.4	—	—	9.4	—	—	—	—	9.4	9.7	—	9.7
5	B	3 Nov.*	26	15.1	—	11.3	—	12.6	—	—	13.9	—	15.8	11.3	—	13.5
6	B	15 Nov.†	23	—	10.9	—	9.3	9.6	10.3†	—	—	—	11.7	10.4	—	10.4
7	C	11 Dec.	20	—	6.5	7.6	—	—	{7.3 7.7	—	—	—	—	—	—	7.3
8	D	13 Jan.	24	10.2	—	9.2	—	12.0	11.3	—	13.2	—	—	—	—	10.9
No. of animals			166	14	14	19	13	26	30	18	18	13	13	19	10.2	—

Each figure in the body of the table represents the mean potassium concentration, in mm/l. of blood, of whole-blood samples taken from groups of three to six cockroaches making up the time-samples indicated.

* Without food, but with water for previous 10 days. † The same animals but with food and water since sampled in series 5.

‡ One animal only. Samples taken during the dark phase are indicated in bold type; onset of darkness at 12.00 hr.

the different batches. In particular, each of the six series of samples taken from batches A and B show a small drop of some 2 mM/l. during the first hour or so of darkness. Batches C and D, which were taken to confirm this observation, did not show such a trend, however.

Because of the different potassium levels of the different batches calculation of mean concentrations for the columns of Table 1 is largely meaningless. The individual values were therefore transformed to percentages of the mean potassium concentration of their respective series. The 166 percentages were then treated as a single set of data and means calculated for the nine columns; these values are illustrated in Fig. 1.

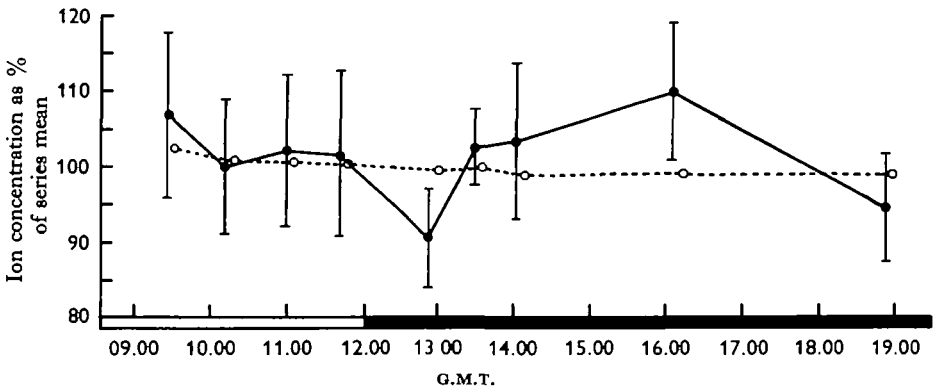


Fig. 1. Changes in the whole-blood potassium and sodium concentration of *Periplaneta* during the period of LD transition and peak locomotor activity. Results are expressed as a percentage of the series mean with the points plotted centrally in the time-span of the samples (see Table 1). Dots and solid line = mean values for potassium concentration (bars = $2 \times$ S.E.); circles and broken line = values for sodium. Abscissa is in hours G.M.T. with the LD transition occurring at 12 noon.

The 12.35–13.10 hr. mean at the onset of darkness shows a fall of about 10% below the values for the preceding hours in the light phase. This fall coincides with the onset of activity, but is not maintained for its duration since the 13.15–13.45 hr. mean shows a reversion to the pre-LD transition level. The 12.35–13.10 hr. value is the only one of the nine which differs significantly from 100% ($P < 0.01$); it is also significantly less than the 09.05–09.45 hr., 13.15–13.45 hr., 16.00–16.15 hr. means and the aggregated mean of 10.40–11.55 hr. ($P < 0.01$ in each case).

The sodium concentration in the blood of the same animals shows no such drop after the LD transition (broken line in Fig. 1), though a steady fall in concentration from 09.00 to 19.00 hr. was observed. The combined means from 09.05 to 11.20 hr. are highly significantly different from the combined means from 13.15 to 16.15 hr. ($P = 0.001$).

These data are open to the criticism that the means may be biased by unequal representation of the different batches, in particular by the values from individuals in batch A which showed the most marked drop after the LD transition. However, where it is possible to sample the data equally from the different batches a probably significant difference is still apparent. Thus the mean of series 1, 5 and 8 at 09.05–09.45 hr. is significantly greater than the mean of these three series at 12.35–13.10 hr. ($P < 0.05$),

though for the difference in series 2, 5 and 8 at 12.35–13.10 hr. and 13.50–14.15 hr. the 5% level of probability is not quite reached ($t = 1.97$ for 24 degrees of freedom, the 5% level being 2.06).

If there is a neuro-physiological effect produced by the apparent drop in potassium concentration, then the timing of the change suggests that it relates to the onset of activity and not to its maintenance. *Periplaneta* kept under strict LD 12:12 show considerable individual variation in the precise phase relationship of their activity to the environmental cycle. There is a range of at least an hour in the exact time of commencement of activity after the onset of darkness, though the timing of a given individual is usually accurate within ± 5 min. from day to day. Population sampling to measure any physiological change at this time will therefore blur the exact dimensions

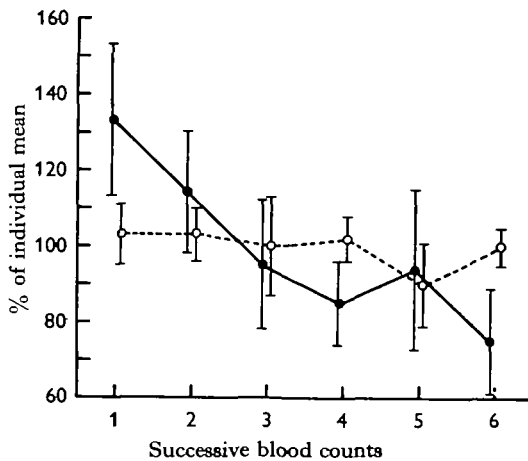


Fig. 2. The effect on the haemocyte count and plasma potassium of taking a series of 6 consecutive blood samples from ten individual *Periplaneta* over a period of 3 weeks. Results are expressed as a % of the individual's mean. Dots and solid line = haemocyte count; circles and broken line = plasma potassium concentration (see text); bars = $2 \times$ s.e.

of the change. If, for example, the drop in potassium at the onset of activity is a transitory phenomenon such a population study will indicate a smaller change than actually occurs in a given individual. An attempt was therefore made to overcome this difficulty by taking series of consecutive samples from single cockroaches whose locomotor activity was being simultaneously monitored in actographs.

It proved possible to take up to five consecutive samples in 10 hr. from a cockroach if it was in good condition. The result of taking the first sample was to raise the haemocyte count dramatically, however, and consequently to cause a rise in the whole-blood potassium concentration (Brady, 1967*d*). This obscured any change in potassium level that might have occurred over the LD transition. To avoid this further difficulty individual animals were sampled at different times every 3rd or 4th day.

At this stage of the study the significance of the haemocyte-contained potassium was realized (Brady, 1967*c*), and the precaution was therefore taken of performing haemocyte counts on all the blood samples before they were analysed. These haemocyte counts indicated that successive sampling caused the cell count to fall, even

when 4 days were allowed to elapse between samples (Fig. 2). However, if the counts were used to correct the whole-blood potassium analyses, on the basis that 10,000 haemocytes/ μ l. of blood represent 0.83 mM K^+ /l. (Brady, 1967*d*), then no change in the plasma concentration was detected for up to six successive samples (Fig. 2). This suggests that successive sampling from single individuals is a legitimate procedure, provided samples are spaced at intervals of a few days, and provided suitable corrections are made to allow for the change in haemocyte density. To avoid any other additive effects the sequence of samples from an individual did not follow the logical course of time across the LD transition but was randomized.

The potassium analyses from the ten *Periplaneta* thus sampled are summarized in Table 2. It is quite clear that no change was detectable in the potassium concentration of either whole-blood or plasma. Similarly, no change was detected in the sodium level. There were no significant differences in the haemocyte counts either, but in view of the results indicated in Fig. 2 this is not entirely surprising.

Table 2. *Summary of potassium levels in successive blood samples taken from ten individual Periplaneta*

(Mean K^+ concentration expressed as a % of the individual animal's mean (\pm s.e.))

	Time of samples in relation to activity record		
	4 to 1 hr. before activity	During 1st hr. of activity	After activity
Whole-blood (haemocytes included)	102 \pm 3	100 \pm 3	99 \pm 3
Plasma (haemocytes subtracted)	101 \pm 3	99 \pm 3	99 \pm 4

Each of the ten cockroaches was maintained in an actograph and sampled 6 times over a period of 3 weeks. The numbers of analyses are roughly equally divided between the 3 columns. The bottom row is estimated on the basis that 10,000 haemocytes/ μ l. represent 0.83 mM K^+ /l. of blood (Brady, 1967*d*).

DISCUSSION

When the cockroaches in batch A were sampled a small fall in the whole-blood potassium concentration was detected coincident with the onset of locomotor activity. A less clearly defined fall was observed in batch B, which was starved before sampling. On the other hand, neither batch C nor D revealed any such change, nor was a change detected by successive sampling from individual animals. The evidence for the existence of a decrease in potassium concentration at the onset of locomotor activity is therefore not entirely clear. However, it is relevant to draw attention to several points which are probably significant.

(1) All four times batch A was analysed a drop in the mean potassium concentration was observed. Though the drop in series 3 occurred in samples collected 10 min. later than the others, this is of no great importance because of the imprecise phase relationships of cockroach activity discussed above. Taking the analyses of batch A alone, the 12.35–13.10 hr. mean (*or* 12.35–13.20 hr. mean to include series 3) is highly significantly less than 100% ($P < 0.001$). These results cannot therefore be discarded as a quirk of chance.

(2) No drop immediately after the LD transition was apparent in the sodium concentration. It therefore appears unlikely that the change in potassium was caused by drinking or feeding at the onset of activity.

(3) It is known that cockroaches respond to the stress of handling, or blood sampling, by showing marked changes in their locomotor pattern, the collection of a blood sample generally resulting in the loss of much of the next activity peak (Brady, 1967*a*). Moreover, these responses are manifest in a form which suggests humoral mediation, so that blood ion changes could possibly be involved, or occur in parallel. If this is so, then the shock of handling may override any subtle circadian changes in the blood of more sensitive individuals.

(4) Batches A and B were cockroaches taken from a stock which derived from *Periplaneta americana* 'wild-caught' at the gardens of the Zoological Society of London 16 months previously, but which had since been kept by the author under controlled conditions in the laboratory. Batch C came from another stock maintained at the Zoological Laboratory, Cambridge, but of indeterminate origin and age. Batch D, and the animals used for successive sampling, came from the London Zoo 18 months after those of batch A. There is no obvious reason for any physiological differences between the two sets of London animals, but they both showed morphological signs of being rather variable hybrids with *P. australasiae*, so there may have been differences in genetic strain. Although it seems improbable that there should be strain differences with respect to anything as fundamental as circadian control, it is not impossible that there might be genetic differences in sensitivity to handling.

(5) Theoretically the successive sampling should have detected any change that the population studies might have obscured. Unfortunately the batch A stock was exhausted by the time these observations were made, and cockroaches from the batch D stock had to be used instead, notwithstanding that these had earlier revealed no blood changes. Furthermore, the handling involved in the successive sampling was considerably more severe than had been used in the population study: it lasted three times longer, included pinioning the animal on its back, and necessitated the collection of two samples of blood (Brady, 1967*d*). If there is any effect of handling and genetic strain it is therefore likely to have been greatest on the successively sampled animals so that the lack of blood ion changes detected by these observations may not be as significant as it appears.

(6) Whereas it is theoretically possible that the fall in potassium could have been caused by a change in the haemocyte density, the data imply that this is unlikely. A mean decrease of some 25,000 cells/ μ l. in the circulating blood would be required to produce the drop of about 2 mM K^+ /l. observed in batch A. Since the mean haemocyte density is about 32,000 cells/ μ l. (Brady, 1967*c*) this seems rather improbable. No change in the haemocytes was observed in the successive samples.

(7) The drop detected in batch A is from about 10 to about 8 mM K^+ /l., but because of the population error this must be a minimal estimate. These figures fit Hoyle's curve (1954) for *Locusta* muscle at a point where a maximum increase of resting potential would be produced by a small decrease in the potassium concentration of the surrounding fluid. The only comparable data for *Periplaneta* are those of Yamasaki & Narahashi (1959) for giant-axon potentials in de-sheathed nerve cord, in which the values are remarkably similar to Hoyle's in the 5–30 mM K^+ /l. range. But, as discussed

by Treherne (1967), the intact central nervous system of the insect exhibits a high degree of ionic regulation so that the observed changes in blood ions would not be expected to cause any immediate axon potential changes centrally. Hoyle's model, however, refers to peripheral phenomena, and there is at present no evidence indicating a comparable level of ionic regulation in insect muscles. There is therefore room to speculate that potassium changes of the order observed in batch A may have a significant peripheral neuro-physiological effect.

CONCLUSION

The initial proposition was that a form of Hoyle's potassium model for the control of locust activity might function in the cockroach as part of the switching mechanism releasing the circadian locomotor peak. No doubt this was a hopeful simplification of a complex physiological system involving many different processes, and in the event only rather uncertain evidence was collected to implicate a potassium-regulated mechanism. Although it would therefore be inappropriate to draw firm conclusions on this point, there does appear to be sufficient evidence to suggest that cockroach blood is a changing ionic environment throughout the day; very clear daily changes in the concentration of both potassium and sodium were detected in some instances. Whether Hoyle's model applies in this case or not, the coincidence of the potassium change with the onset of peak activity appears to be *prima facie* evidence for the existence of some form of ionic regulation in the locomotor rhythm.

SUMMARY

1. A study was made of the concentration of potassium and sodium in the blood of *Periplaneta americana* to determine whether daily fluctuations occur which might imply ionic control of the circadian locomotor activity rhythm.
2. Analyses were carried out on 166 blood samples taken at different times of day. A fall of about 10% occurred in the potassium concentration during the first hour of darkness; there was also a gradual decline of 2% in the sodium concentration.
3. However, series of six successive blood samples taken over a period of 3 weeks from ten individual cockroaches revealed no daily change in the level of potassium, sodium or haemocyte density.
4. No conclusions are drawn on the activity control aspect, but the results do suggest that daily changes may occur in cockroach blood ions.

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REFERENCES

- BRADY, J. (1967*a*). Control of the circadian rhythm of activity in the cockroach. I. The role of the corpora cardiaca, brain and stress. *J. exp. Biol.* **47**, 153-63.
- BRADY, J. (1967*b*). Control of the circadian rhythm of activity in the cockroach. II. The role of the sub-oesophageal ganglion and ventral nerve cord. *J. exp. Biol.* **47**, 165-78.
- BRADY, J. (1967*c*). Haemocytes and the measurement of potassium in insect blood. *Nature, Lond.* **215**, 96-7.
- BRADY, J. (1967*d*). The relationship between blood ions and blood-cell density in insects. *J. exp. Biol.* **47**, 313-26.
- ELLIS, P. E. & HOYLE, G. (1954). A physiological interpretation of the marching of hoppers of the African migratory locust (*Locusta migratoria migratorioides* R. & F.). *J. exp. Biol.* **31**, 271-9.
- HARKER, J. E. (1964). *The Physiology of Diurnal Rhythms*. Cambridge University Press.
- HOYLE, G. (1954). Changes in the blood potassium concentration of the African migratory locust (*Locusta migratoria migratorioides* R. & F.) during food deprivation, and the effect on neuromuscular activity. *J. exp. Biol.* **31**, 260-70.
- PICHON, Y. & BOISTEL, J. (1963). Modifications of the ionic content of the haemolymph and of the activity of *Periplaneta americana* in relation to diet. *J. Insect Physiol.* **9**, 887-91.
- ROBERTS, S. K. DE F. (1966). Circadian activity rhythms in cockroaches. III. The role of endocrine and neural factors. *J. cell. Physiol.* **67**, 473-86.
- TREHERNE, J. E. (1967). Axonal function and ionic regulation in insect central nervous tissues. In *Insects and Physiology*, pp. 175-88. Eds. J. W. L. Beament and J. E. Treherne. Edinburgh and London: Oliver and Boyd.
- YAMASAKI, T. & NARAHASHI, T. (1959). The effects of potassium and sodium ions on the resting and action potentials of the cockroach giant axon. *J. Insect Physiol.* **3**, 146-58.